

Male-Biased Sex Ratios of Fish Embryos near a Pulp Mill: Temporary Recovery after a Short-Term Shutdown

D. G. Joakim Larsson and Lars Förlin

Department of Zoology/Zoophysiology, Göteborg University, Göteborg, Sweden

In a previous study we showed that broods from the viviparous eelpout (*Zoarces viviparus*) were significantly male biased in 1998 in the vicinity of a large kraft pulp mill on the Swedish Baltic coast. One suggested hypothesis was that masculinizing compounds in the effluent were affecting gonadal differentiation of the embryos, resulting in skewed sex ratios. In this article, we present further evidence for a causal relationship between the exposure to the effluent and the male-biased sex ratios. Analyses of historical samples showed that the eelpout produced male-biased broods close to the mill in 1997 in addition to 1998. During 1999, the mill was shut down for 17 days, coinciding with the period when the gonads of the eelpout embryos differentiate. Subsequently, in the fall of 1999, the sex ratios were no longer male biased; however, the following year (2000), a significant male bias reappeared. Investigations at 13 sites for up to 4 years showed a relatively stable sex ratio around 50/50, with the exceptions by the mill and with few observations of deviating ratios at other sites. Several reports document endocrine disturbances in fish near pulp and paper mills, including the expression of male secondary sex characters in female fish. The repeatedly identified male bias at the investigated mill, the normalization after mill shutdown, and the reappearance the following year indicate that pulp mill effluents also can affect sex ratios of nearby fish. **Key words:** androgenic, endocrine disruptors, gonadal differentiation, masculinization, ovary, testis. *Environ Health Perspect* 110:739–742 (2002). [Online 11 June 2002] Figure 1 was corrected on 29 July 2002.

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Pulp mills release large amounts of complex effluents more or less continuously into our aquatic environment. In general, the toxicity of these effluents has decreased substantially during the past decades through more closed systems, introduction of nonchlorine bleaching, and more efficient secondary treatment of the effluents (1). Despite these improvements, there is ample evidence for disturbed benthic algal and invertebrate communities in the recipients (1–3). Effects on the hepatic cytochrome P450 system of fish are also regularly observed, as is interference with their reproductive system (4–11), including depressions of circulating steroid hormones, delayed maturation, smaller gonad size, and altered expression of secondary sex characters. In the United States and New Zealand, female mosquitofish (*Gambusia* sp.) exposed to pulp and paper mill effluent, both in the field and in the laboratory, develop an elongated anal fin resembling the male gonopodium (12–15). Overexpression of male secondary sex characters has also been found in eels (*Anguilla rostrata*) (16) and white suckers (*Catostomus commersoni*) (17) exposed to paper/pulp mill effluents. Because androgens are known to induce male secondary sex characters in many fishes, the hypothesis was generated that the effluents contain androgenic compounds (12). In addition to the effects on secondary sex characters, androgens (and estrogens) can direct gonadal sex toward male (or female)

development in many fish species if applied at a critical time at or just before the onset of gonadal differentiation (18). Exposure experiments with eggs from fathead minnows (*Pimephales promelas*) and a diluted Canadian bleach kraft mill effluent produced not only a greater proportion of adult fish expressing male secondary sex characters, but also a greater proportion of males as judged by the presence of testis (19). These findings led us to investigate the sex ratios of embryos of the viviparous eelpout (*Zoarces viviparus*) in the vicinity of a large kraft pulp mill on the Swedish Baltic coast (20). The eelpout undergo gonadal differentiation as embryos and are simple to sex before birth (20). Thus, in contrast to most oviparous species, accurate sex ratios from individual broods are easy to obtain from the field. The sex ratios of embryos sampled in 1998 were significantly male biased (42% females) close to the mill, whereas the normal sex ratio appeared to be around 50% females at all studied reference sites (20). We therefore hypothesized that masculinizing components in the effluent were causing the male-biased broods.

Our aim in the present study was to investigate further the link between pulp mill effluent exposure and male-biased sex ratios of the eelpout. We therefore investigated the consistency of the response over 4 years at the mill and at several reference sites. We also included sites close to other industrial activities to investigate the specificity of the cause. We

discuss the data in context of the classic criteria used in ecoepidemiologic research (21).

Materials and Methods

Sex ratio analyses. The embryos of the viviparous eelpout initiate gonadal differentiation in late September or early October while still in the mother's ovary (20). The sex of the embryos can be distinguished easily and accurately before birth by morphologic differences between the embryonic testes and ovary (20). We performed sex ratio analyses *in situ* on Bouin's-fixed embryos sampled from late October to mid November as described previously (20). In total, we sexed 15,895 embryos from 425 adult females. We treated all animals in accordance with the requirements of the ethical committee in Gothenburg, Sweden.

Sampling sites. The investigated mill is a kraft pulp mill located on the Swedish Baltic coast. The mill is using total chlorine-free bleaching technology (bleaching process Q/OP/ZQ/PO), and it alternates between softwood and hardwood pulp production. Activated sludge treatment was introduced during the summer of 1998, replacing aerated lagoons for effluent treatment. The effluent earlier entered the Baltic Sea via a 5-km-long tube equipped with a 1.5-km-long diffuser. The extension of the effluent plume has been described elsewhere (22). In 1997, the lid covering the end of the diffuser was open, probably resulting in a somewhat different plume, and in 1999, a 250-m-long diffuser permanently replaced the old one (23). From 20 September to 6 October

Address correspondence to J. Larsson, Department of Physiology/Endocrinology, Sahlgrenska Academy, Göteborg University, Box 434, SE-405 30, Göteborg, Sweden. Telephone: +46-31-7733589. Fax: +46-31-7733531. E-mail: joakim.larsson@fysiologi.gu.se

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1999, the mill instituted a planned complete shutdown of its pulp production to prepare for an increased production capacity from 550,000 to 800,000 metric tons of pulp per year. Over a few days before and after this stop, production gradually decreased and increased, respectively. This period comprised the time just before and during the expected onset of morphologic differentiation of the embryonic gonads of the eelpout (20). There have been shutdowns during other years as well, but long before or after the onset of gonadal differentiation of the eelpout. During a shutdown, organic material usually leaks continuously from the mill, but the character of the effluent is probably much different from that of normal production (23).

A river with a long history of organochlorine and heavy metal pollution (24) enters the Baltic Sea approximately 10 km north of the pulp mill outfall. In 1999 and 2000, the investigation was expanded to include an additional site 10 km north of the mill outfall, to evaluate if water from this river could have caused the previously observed male bias. To investigate whether other types of stressors could affect the sex ratios, we sampled eelpout in 1999 on the Swedish West coast in the vicinity (1–2 km) of an oil refinery in Brofjorden, close to the effluent outfall from Stenungsund petrochemical industries (< 1 km) and two sites in the Göta River Estuary. The latter two receive a mixture of sewage and industrial effluents from the Gothenburg region and are located 1 and 3 km downstream from the outfall of the local sewage treatment plant. The reference sites have previously been used in regional environmental monitoring programs/physiologic studies of eelpout and perch (*Perca fluviatilis*) (25–31).

Statistical analyses. For each year, we pooled sex ratios from the preselected reference sites and compared them with sex ratios of each of the other sites using Student's *t*-tests (no Bonferroni correction). When results from residual analyses did not meet the assumptions of parametric tests, we performed randomization analyses without replacement according to Manly (32).

Results

In 1997 the embryonic sex ratios were significantly male biased (45.5% females) 1.7 km north of the mill compared with the pooled reference sites ($p = 0.0015$), whereas sex ratios at the site 1.2 km south of the mill showed a similar trend but were not significantly different from the reference sites ($p = 0.057$; Figure 1). Further south in the discharge gradient the sex ratios approached reference levels. This pattern was very similar to that previously reported for 1998 when the two closest sites were significantly male biased (42.2% and 46.1%) compared with the four control sites

($p = 0.0005$ and $p = 0.042$), and a similar gradual normalization was apparent as moving south from the mill (Figure 1) (20).

In 1999, the year when the mill had a planned shutdown period (see "Materials and Methods"), we found no significant differences between the two pooled preselected reference sites and any other site. We found no trends suggesting a male bias at the site closest to the river mouth (10 km north) or closer to the mill (Figure 1). On the other hand, the northernmost reference site showed a significantly lower sex ratio compared with 1998 ($p = 0.046$).

In 2000, the sex ratios at the site closest north of the mill was again male biased (38.7%) compared with the pooled references ($p = 0.0004$; Figure 1). Also, two of the three sites south of the outfall were male biased (41.3%, 41.7%; $p = 0.020$, 0.014). The site closest south of the outfall (47.0%) and the site closest to the river mouth (48.0%) did not differ significantly from the pooled references ($p = 0.94$ and $p = 0.71$, respectively).

In 1999, we studied the specificity of the sex ratio deviations by sampling fish from a reference site in Skagerrak (same as in 1998), an oil refinery, a petrochemical industry, and two sites in the Göta River Estuary, all on the Swedish west coast. The sex ratios were close to 50/50 at all sites and no site differed significantly from the control site (Figure 2).

Discussion

We have shown that sex ratios of eelpout broods in the vicinity of a pulp mill were male biased in 1997, 1998, and 2000. In 1999, the mill shut down at a time when the embryos normally undergo gonadal differentiation (20). When we sampled the eelpout later in the fall, the sex ratios were normalized; however, the following year the eelpout by the mill again produced male-biased broods. We hypothesize that the effluent from the mill is causing the male bias, and a reduced exposure to the effluent during the period of gonadal differentiation in 1999 allowed the eelpout to produce broods with normal sex ratios.

Fox (21) has defined a set of ecoepidemiologic criteria to be used to evaluate whether a causal relationship is likely to be present between an environmental stressor and an observed response. These include probability, time order, strength, specificity and consistency of association, predictive performance, and coherence (theoretical, factual, biologic, and dose-response). Below, we have tried to apply these criteria to the male-biased sex ratios by the Swedish mill.

The probability of a causal relationship refers to statistical significance. Indeed, over 3 years, a statistically significant male bias developed at one or both of the two sites closest to the mill compared with the preselected reference sites. A statistically

significant correlation may imply causality, but it is a strong argument only when it can be backed up by other information. The time order is such a criterion, which ideally should include not only appearance of the effect after the stressor is introduced, but also disappearance of the effect when the stressor is removed and reoccurrence when the stressor is introduced again. The investigated mill has been releasing effluent for several decades, although exposure may have varied with different processes, production, and alternating currents in the area (22). No historical samples of eelpout embryos are available to investigate sex ratios before the mill was built. However, the temporary shutdown in 1999 of the mill during the onset of the gonadal differentiation of the eelpout opened up an unusual opportunity to demonstrate the disappearance of the effect after a presumably reduced exposure. A previous study at a large pulp mill in Jackfish Bay, Lake Superior, Canada, showed that circulating 11-ketotestosterone levels of male white suckers recovered within 2 weeks after a maintenance shutdown (6). This suggested that the fish were able to approach a normal physiology in a relatively short time, implying that at least some of the endocrine

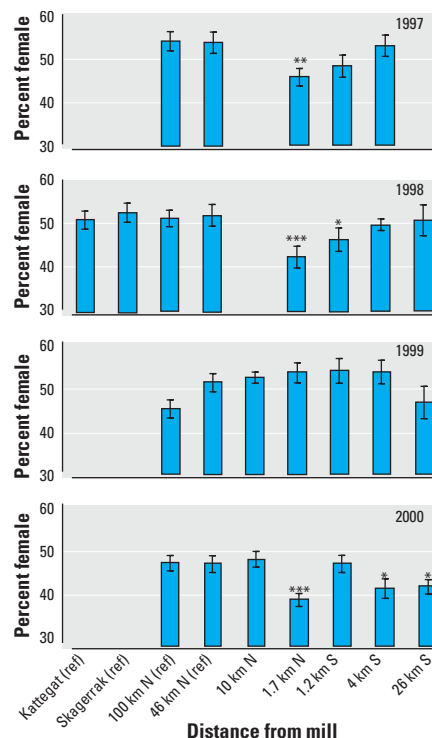


Figure 1. Embryonic sex ratios of eelpout broods (mean ± SEM) sampled at reference sites (ref) or at different distances [north (N) or south (S)] from a pulp mill on the Swedish Baltic coast from 1997 to 2000.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significant difference from the pooled reference sites within the same year. Previously published data from 1998 (20) have been included with permission from the Society of Environmental Toxicology and Chemistry.

disruptors were relatively rapidly metabolized and/or excreted by the fish. The recovery to normal sex ratios of the eelpout after the 17-day pulp mill shutdown and the reappearance of the male bias the following year agree with the observations at Jackfish Bay.

The strength of association refers to the relative occurrence of the effect when the stressor is present compared with when it is not. Over the 4 years of the investigations, we had one observation of significantly male-biased sex ratios at a presumably clean site (100 km north, 1999) and one observation at a site where the exposure levels are expected to be very low or zero (26 km south, 2000), revealing that eelpout sex ratios may show some degree of natural, or at least unexplained, variation. However, at the two sites closest to the mill, four of six possible observations (excluding the year of the shutdown) were significantly male biased, and one observation in 1997 was very close to significance ($p = 0.057$). This represents a much higher relative occurrence.

We addressed the specificity of the potential cause by sampling eelpout exposed to other potential stressors—that is, near an oil refinery; near a petrochemical industry; near a river with organochlorine and heavy metal pollution; and at two sites with a mixed exposure situation. At none of these sites were the sex ratios significantly skewed. The disappearance of the effect by the mill in 1999 also gives some information about the specificity, suggesting no causal relationship between any permanent conditions at the two sites closest to the mill and the skewed sex ratios. Such conditions, present throughout the investigation, include alterations of the benthic fauna and flora in the close recipient. The most obvious alteration is a strong reduction in *Fucus vesiculosus*, the dominating large brown algae in the Baltic, hosting a whole ecosystem of small animals (29,33,34).

Regarding the consistency of association upon replication, we demonstrated the male bias over 3 years. We performed the actual sex ratio analyses with different personnel

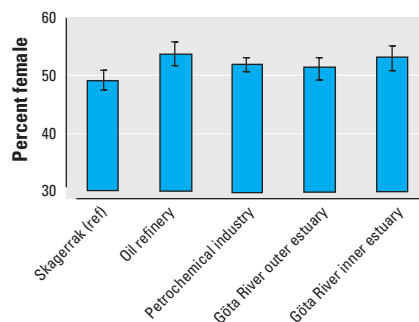


Figure 2. Embryonic sex ratios of eelpout broods (mean ± SEM) sampled in 1999 at a reference site in Skagerrak or in the proximity of different industrial activities (see “Materials and Methods” for details).

each year, showing independence of the examiner. A lab study exists (19), but to our knowledge no field study other than at this mill (20, present study), has yet shown a change in sex ratio in fish associated with pulp mill effluent exposure. This warrants further investigations at this and other mills, ideally with more species. However, the possibly related effects of pulp and paper mills on male secondary sex characters in different fish species have been identified repeatedly at several times and locations (12,14–17).

In our previously published study, we presented the first data on the male bias close to the mill (20). In this follow-up study, we had the opportunity to test the predictive performance of our hypothesis. At all times, the observed sex ratios by the mill have been similar to the predictions from the hypothesis.

There is clear coherence between skewed sex ratios and pulp mill effluent exposure. Two research groups recently identified the presence of androgen receptor ligands in the Fenholloway River, which is heavily contaminated by paper mill effluent (35,36). Androstenedione, a moderately strong steroidal androgen and also a precursor for more potent androgens, was found in a concentration of 0.14 nM (35). Female mosquitofish in the Fenholloway River display male secondary sex characters (35,36). Thus, androstenedione is a good candidate for causing the observed masculinizations of mosquitofish; but the relative contribution of other steroid receptor ligands remains to be evaluated. Hewitt et al. (37) showed that fish exposed to kraft pulp mill effluent accumulate androgen and estrogen receptor ligands in their livers. Taking the findings of induced male secondary sex characters into consideration, we have extensive evidence for the presence of masculinizing agents in pulp and paper mill effluents. Also, a physiologic mechanism for androgen exposure leading to male-biased sex ratios has been established (18). For all 3 years when we observed an effect in the recipients of the effluent, the north site closest to the mill showed the lowest male-to-female sex ratio. Also, we found the strongest male bias after the increase in production capacity in 2000, and two sites farther south in the gradient were also male biased. These observations could represent a dose–response relationship, albeit the data are not entirely clear. For example, the site north of the mill (1.7 km) consistently displayed a stronger male bias compared with the site immediately south of the mill, which is closer to the outfall (1.2 km), and in 2000 the sex ratio was not male biased at the closest site south of the outfall. Because the gonadal differentiation of fish, in general, is sensitive to hormones only during a short time window, the direction of the effluent plume at a particular time point may be of importance. The normal-

ization of the sex ratios by the mill after the 17-day shutdown in 1999 supports the hypothesis that the male bias is not caused by chemicals accumulated over long periods of time.

As an alternative hypothesis, we have earlier discussed exposure to potentially increased temperatures near the mill (20). As is true for exogenous hormonal exposure, altered environmental temperatures have the potential to affect gonadal differentiation in many fish species (38). At present, we have little support for a significant temperature gradient around the mill outfall (29) but ample data suggest the presence of endocrine-disrupting compounds in pulp mill effluents. To address further the temperature hypothesis, we would like to gather more detailed temperature data in the proximity of the outfall and at reference sites. Another alternative hypothesis is that the sex ratios are skewed near the mill because of parental “choice.” One of the most important factors determining the fitness of an individual is the sex ratio of its offspring. Hence, an ability to adjust offspring sex ratios to certain conditions has developed in many animal species (39). Some common cues related to offspring sex ratios in different species include size of mother, number of embryos, and the size of the offspring. These, however, are not important predictors of offspring sex ratios in the eelpout (20). Nor is sex-selective early embryonic mortality a mechanism by which the observed male bias is brought about (20). A sex ratio of approximately 50% female embryos appears to be the norm for eelpout at a variety of sites in the Baltic, Kattegat, and Skagerrak. We observed skewed sex ratios only twice in the present study, except at the three sites closest to the mill. Under some natural environmental conditions eelpout may produce broods with skewed sex ratios. Although it would be very coincidental, it cannot be ruled out that such conditions were present by the mill in 1997, 1998, and 2000 but not in 1999.

Field data can rarely supply finite evidence for a causal relationship; however, by applying the above criteria, the probability of causality can be judged. Our interpretation is that a substantial amount of data must be ignored to accept the null hypothesis, that no causal relationship exists between exposure to the effluent from the mill and the male bias of the sex ratios. Although skewed sex ratios were found in fathead minnows exposed to another kraft pulp mill effluent in a laboratory experiment (19), well-performed dose–response exposures with effluent from the presently investigated mill would provide additional information. We have had some success with spawning eelpout in captivity, overcoming some of the difficulties in successfully catching, transporting, and holding

wild cold-water marine fish like the eelpout for long periods of time in captivity. However, to expose experimental groups continuously to diluted effluent water, while supplying appropriate conditions for maturation, spawning, and pregnancy, is a major practical challenge. We have exposed female guppies (*Poecilia reticulata*), a smaller and more "user-friendly" fish species, to diluted effluent from the investigated mill. Exposed females developed a male-like coloration, suggesting that masculinizing compounds are present in the effluent. However, we found no effects on the anal fin morphology or the apparent offspring sex ratio (40). Both primary and secondary sex characters can be affected by androgen treatment in poeciliids, but the lack of understanding of the detailed mechanisms involved is presently preventing a full interpretation of these results. Preliminary results from experiments on fractionated effluent from the investigated mill showed that components binding to fish gonadal androgen receptors indeed are present in the water (41). Also, spiggin, an androgen-inducible protein in the stickleback (*Gasterosteus aculeatus*), was induced after exposure *in vivo* to the effluent (42). From the available field and experimental data, we conclude that the best available hypothesis to date is that compounds in the pulp mill effluent are causing the male bias of the eelpout broods.

Offspring sex ratios are crucial for individual fitness and population recruitment potential; therefore, any effect of pollution on sex ratios should be carefully evaluated. The adult eelpout catches per effort were not apparently smaller by the mill than at other investigated sites (43). However, population effects of a decreased recruitment potential in certain situations likely are less important for adult population size than are variations in food availability, predator abundance, shelter possibilities, migration, and so forth. The issues of how sex ratio changes of the eelpout can affect population and ecosystem dynamics need to be further addressed (20). Although not yet investigated, it is quite plausible that other fish species in the recipient could be directly affected. In a more restricted environment, such as in lakes or rivers, population effects are more likely to occur, whereas compensatory migration may be important on the coast over longer times. Several laboratories worldwide, including ours, are presently working on the identification of endocrine-disrupting substances in pulp and paper mill effluents (35–37,41,44). We hope the results from these studies will eventually enable an evaluation of possible technology adjustments in relation to the severity of the potential environmental consequences.

REFERENCES AND NOTES

- Sandström O, Förlin L, Grahm O, Landner L, Larsson Å, Lindesjö E. Environmental Impact of Pulp and Paper Mill Effluents. A Strategy for Future Environmental Risk Assessment. Report 4785. Stockholm:Swedish Environmental Protection Agency, 1997.
- Sundelin B, Eriksson A-K. Malformation in embryos of the deposit-feeding amphipod *Monoporeia affinis* in the Baltic Sea. *Mar Ecol Prog Ser* 171:165–180 (1998).
- Wikström S, Kautsky L, Malm T. The effects of chlorine free pulp mill effluents on reproduction and grazing interactions in Baltic Sea *Fucus vesiculosus* L. *Ophelia* 53:173–179 (2000).
- McMaster, Van der Kraak GJ, Portt CB, Munkittrick KR, Sibley PK, Smith IR, Dixon DG. Changes in hepatic mixed function oxygenase (MFO) activity, plasma steroid levels and age at maturity of a white sucker (*Catostomus commersoni*) population exposed to bleached kraft pulp mill effluent. *Aquat Toxicol* 21:199–218 (1991).
- Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. Exposure to bleached kraft mill effluents disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol Appl Pharmacol* 115:224–233 (1992).
- Munkittrick KR, Van der Kraak GL, McMaster ME, Portt CB. Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environ Toxicol Chem* 11:1427–1439 (1992).
- Munkittrick KR, Van der Kraak GJ, McMaster ME, Portt CB, Van den Heuvel MR, Servos MR. Survey of receiving water environmental impacts associated with discharges from pulp mills. II. Gonad sex, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. *Environ Toxicol Chem* 13:1089–1101 (1994).
- Förlin L, Andersson T, Balk L, Larsson Å. Biochemical and physiological effects of bleached pulp mill effluents in fish. *Ecotoxicol Environ Saf* 30:164–170 (1995).
- McMaster ME, Van der Kraak GJ, Munkittrick KR. An epidemiological evaluation of the biochemical basis for steroid hormonal depression in fish exposed to industrial wastes. *J Great Lakes Res* 22:153–171 (1996).
- Munkittrick KR, Servos MR, Carey JH, Van der Kraak GJ. Environmental impact of pulp and paper waste water: evidence for a reduction at North American pulp mills since 1992. *Water Sci Technol* 35:329–338 (1997).
- Karels A, Soimasu M, Oikari A. Effects of pulp and paper mill effluents on reproduction, bile conjugates and liver MFO (mixed function oxygenase) activity in fish at southern Lake Saima, Finland. *Water Sci Technol* 40:109–114 (1999).
- Howell WM, Black DA, Bortone SA. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally induced masculinization. *Copeia* 4:676–681 (1980).
- Drysdale DT, Bortone SA. Laboratory induction of intersexuality in the mosquitofish, *Gambusia affinis*, using paper mill effluent. *Bull Environ Contam Toxicol* 43:611–617 (1989).
- Cody RP, Bortone SA. Masculinization of mosquitofish as an indicator of exposure to kraft mill effluent. *Bull Environ Contam Toxicol* 58:429–436 (1997).
- Ellis RJ, van den Heuvel MR, Stuthridge TR, McCarthy LH, Ling N, Hogg ID, Dietrich DR. "Androgenic responses" in two fish species following exposure to a New Zealand pulp and paper mill effluent. *Toxicologist* 60:162 (2001).
- Caruso JH, Suttus RD, Gunning GE. Abnormal expression of secondary sex characteristics in a population of *Anguilla rostrata* (Pisces: Anguillidae) from a dark coloured Florida stream. *Copeia* 4:1077–1079 (1988).
- Munkittrick KR, McMaster ME, Servos MR, Van der Kraak GJ. Secondary sex characteristics and gonadal size in white sucker (*Catostomus commersoni*) during modernization at a pulp mill. Proceedings from the 6th International Symposium on Reproductive Physiology of Fish. Bergen, Norway, 4–9 July. Bergen:University of Bergen, 1999; 383.
- Hunter GA, Donaldson EM. Hormonal sex control and its application to fish culture. In: *Fish Physiology*, Vol 9 (Hoar WS, Randall DJ, Donaldson EM, eds). New York: Academic Press, 1983; 223–303.
- Kovacs TG, Gibbons JS, Tremblay LA, O'Connor BI, Martel PH, Voss RH. Effects of a secondary-treated bleached kraft mill effluent on aquatic organisms as assessed by short- and long-term laboratory test. *Ecotoxicol Environ Saf* 31:7–22 (1995).
- Larsson DGJ, Hällman H, Förlin L. More male fish embryos near a pulp mill. *Environ Toxicol Chem* 19:2911–2917 (2000).
- Fox GA. Practical causal interference for ecotoxicologists. *J Toxicol Environ Health* 33:359–373 (1991).
- Landner L, Grahm O, Härdig J, Lehtinen K-J, Monfelt C, Tana J. A field study of environmental impacts at a bleached kraft pulp mill site on the Baltic sea coast. *Exotoxicol Environ Saf* 27:128–157 (1994).
- Löfblad R. Personal communication.
- Gullbring P, Hammar T, Helgö A, Troedsson B, Hansson K, Hansson F. Remediation of PCB-contaminated sediments in Lake Jämsjön: investigations, considerations and remedial actions. *Ambio* 5:374–384 (1998).
- Förlin L, Celander M. Induction of cytochrome P450 1A in teleosts: environmental monitoring in Swedish fresh, brackish and marine waters. *Aquat Toxicol* 26:41–56 (1993).
- Balk L, Andersson T, Förlin L, Söderström M, Larsson Å. Indications of regional and large-scale biological effects caused by bleached pulp mill effluents. *Chemosphere* 27:631–650. (1993).
- Balk L, Larsson Å, Förlin L. Baseline studies of biomarkers in the feral female perch (*Perca fluviatilis*) as tools in biological monitoring of anthropogenic substances. *Marine Environ Res* 42:203–208 (1996).
- Vetema M, Förlin L, Sandström O. Chemical industry effluent impacts on reproduction and biochemistry in a North Sea population of viviparous blenny (*Zoarces viviparus*). *J Aquat Ecosyst Stress Recov* 6:33–41 (1997).
- Anonymous. Samordnad kustvattenkontroll i Kalmar län. Årsrapport 1998 [in Swedish]. Kalmar, Sweden:Kalmar läns kustvattenkommitté, 1998.
- Ronisz D, Larsson DGJ, Förlin L. Seasonal variations in the activities of selected hepatic biotransformation and antioxidant enzymes in eelpout (*Zoarces viviparus*). *Comp Biochem Physiol* 124:271–279 (1999).
- Larsson DGJ, Mayer I, Hyllner S-J, Förlin L. Seasonal variations of vitelline envelope proteins, vitellogenin and reproductive steroids in eelpout (*Zoarces viviparus*). *Gen Comp Endocrinol* 125:184–196.
- Manly BFJ. Randomization and Monte Carlo Methods in Biology. London: Chapman & Hall, 1991.
- Anonymous. Samordnad kustvattenkontroll i Kalmar län. Årsrapport 1999. Kalmar läns kustvattenkommitté [in Swedish]. Kalmar, Sweden: 1999.
- Anonymous. Samordnad kustvattenkontroll i Kalmar län. Årsrapport 2000. Kalmar läns kustvattenkommitté [in Swedish]. Kalmar, Sweden: 2000.
- Jenkins RJ, Angus RA, McNatt H, Howell WM, Kempainen JA, Kirk M, Wilson EM. Identification of androstenedione in a river containing paper mill effluent. *Environ Toxicol Chem* 20:1325–1331 (2001).
- Parks LG, Lambright CS, Orlando EF, Guillette LJ, Ankley GT, Gray LE Jr. Masculinization of female mosquitofish in kraft mill effluent contaminated Fenholloway river is associated with androgen receptor agonistic activity. *Toxicol Sci* 62:257–267 (2001).
- Hewitt M, Parrot JL, Wells KL, Calp MK, Biddiscombe S, McMaster M, Munkittrick KR, Van der Kraak G. Characteristics of ligands for the Ah receptor and sex steroid receptors in hepatic tissues of fish exposed to bleached kraft mill effluent. *Environ Sci Technol* 34:4327–4334 (2000).
- Strüssman CA, Patiño R. Sex determination, environmental. In: *Encyclopedia of Reproduction*, Vol 4 (Knobil E, Neill JD, ed). San Diego: Academic Press, 1998;402–409.
- Ericsson RJ, Ericsson SA. Sex ratios. In: *Encyclopedia of Reproduction*, Vol 4 (Knobil E, Neill JD, eds). San Diego, CA:Academic Press, 1998;431–427.
- Larsson DGJ, Kinnberg K, Sturve J, Stephensen E, Skön M, Förlin L. Studies of masculinization, detoxification and oxidative stress responses in guppies (*Poecilia reticulata*) exposed to effluent from a pulp mill. *Ecotoxicol Environ Saf* 52:13–20.
- Larsson DGJ, Adolfsson-Erici M, Thomas P. Unpublished data.
- Katsiadaki I, Scott AP, Hurst MR, Matthiessen P, Mayer I. A novel method based on ELISA of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein, for the detection of environmental androgens. *Environ Toxicol Chem* (in press).
- Andersson J. Department of Fisheries. Personal communication.
- Kiparissis Y, Hughes R, Metcalfe CD, Ternes T. Identification of the isoflavonoid genistein in bleached kraft mill effluent. *Environ Sci Technol* 35:2423–2427 (2000).